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EXPERIMENTAL PAPER

Impact of *Curcuma longa* extract on the expression level of brain transporters in *in vivo* model

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Summary

Introduction: Blood brain barrier and multidrug resistance phenomenon are subjects of many investigations. Mainly, because of their functions in protecting the central nervous system (CNS) by blocking the delivery of toxic substances to the brain. This special function has some disadvantages, like drug delivery to the brain in neurodegenerative diseases.

Objective: The aim of this study was to examine how natural and synthetic substances affect the expression levels of genes (Mdr1a, Mdr1b, Mrp1, Mrp2, Oatp1a4, Oatp1a5 and Oatp1c1) that encode transporters in the blood-brain barrier.

Methods: cDNA was synthesized from total RNA isolated from rat hippocampus. The expression level of genes was determined using real-time PCR (RT-PCR) method.

Results: Our findings showed that verapamil, as a synthetic substance, caused the greatest reduction of mRNA level of genes studied. The standardized extract of *Curcuma longa* reduced the expression level for Mrp1 and Mrp2, whereas the increase of mRNA level was observed for Mdr1b, Oatp1a5 and Oatp1c1.

Conclusions: These results suggests that herbal extracts may play an important role in overcoming the blood brain barrier during pharmacotherapy.

Key words: ***blood brain barrier, Curcuma longa, expression level, natural and synthetic substances***

Słowa kluczowe: ***bariera krew-mózg, Curcuma longa, poziom ekspresji, substancje naturalne i syntetyczne***

INTRODUCTION

Brain diseases might take various forms, such as infections, trauma, stroke, seizures and tumors. Brain inflammations can be caused by viral and bacterial infections [1]. Epilepsy is a brain disease categorized as a seizure condition, which is characterized by abnormal brain's electrical activity [2]. Trauma is a brain injury that may cause a temporary or a permanent disturbance in brain function [3]. Tumors are abnormal cells that form tissues and grow inside the brain. Whether malignant or benign, tumors are very dangerous due to the pressure they exert on an unchanged brain tissue [4]. Finally, stroke is a brain disease connected with the condition of blood vessels. During stroke, blood and oxygen flow are suddenly disturbed. Areas of brain that weren't properly supplied by blood and oxygen might be permanently damaged. Body parts which are controlled by those damaged brain areas might no longer work correctly – or even at all [5].

Even though the forms of brain diseases differ in etiology or symptoms, most of them lead to cell death [6-8]. Due to the great importance of brain, such as neural signaling within central nervous system (CNS), it requires microenvironment that would be highly controlled. There are three forms of protection between blood and CNS: the blood barrier (BBB), the blood-CSF barrier and also the arachnoid barrier [9]. The blood barrier is a selective membrane. BBB creates a chemical and physical barrier between blood and brain parenchyma. As to specialized cells and transporters that the BBB has, it can regulate the environment of CNS [6]. Thanks to those regulations, CNS may function properly [10]. The homeostasis of CNS might be changed by morphological or functional disturbances that can result in brain damage [11, 12]. As the BBB's main role is to stop the transports of majority of substances to the brain, tightness of this barrier is variable. The BBB contains numerous transporters and channels that allow to be transported nutrients needed to proper functioning of the brain [6]. The BBB also contains ion transporters and channels that appear to be promising as a therapeutic path to be challenged.

There are many studies that explore usefulness of transporters as a drug deliver paths in brain diseases [11]. Transporters expressed by CNS may be divided into two types: efflux and nutrient. Firstly, the efflux transporters (e.g. MDR1, BCRP, MRPs) use the ATP's hydrolysis to transport their substrates up the concentration gradient. While the nutrient transporters simplify the transport of the specific substances down the concentration gradient (e.g. slc2a1, slc16a1, slc7a1, slc7a). Despite the

fact that those transporters mainly provide substances to the brain, some of them are removing waste and toxic products from the brain. In brain diseases, all of the protective functions of the BBB are weaken [10, 13, 14].

For many years, neurological diseases were only diagnosed and the treatment was almost impossible. Nowadays, the neurology itself blossoms and it gives a hope to find new drugs and ways to deliver them [8, 15]. It is believed that new drug programmes should consider the special features of the BBB [10]. Unfortunately, most of clinical substances commonly used in brain diseases provide narrow improvement and are accompanied by side effects. Despite all difficulties, treatment of CNS diseases still remains a major goal for scientists [16]. New studies show how interesting is the effect of the substances of herbal origin on the expression of transporter gene conditioning, the blood brain barrier and phenomenon of multidrug resistance (MDR). Most of studies focus on P-gp expression and the impact of synthetic and herbal substances. Verapamil is one of the synthetic substances taken into consideration as a P-gp inhibitor. Unfortunately, due to cardiac toxicity, verapamil cannot be allowed in practice. However, the inhibitors of herbal origin seem to have less side effects on normal tissues than synthetic substances. Furthermore, some flavonoid compounds, such as kaempferol, daidzein, quercetin, genistein, may decrease the P-gp pump's activity. The mechanism is based on the reduction of the resistance of selected cell lines to vinblastin. Curcumin (from rhizomes of *Curcuma longa* L., *Zingiberaceae*), for example, is believed to decrease the P-gp's activity [17, 18].

Consequently, the main aim of this paper was to analyze the effects of synthetic and herbal substances, which might be important in blocking the function of transporters in blood-brain barrier.

EXPERIMENTAL

Study design

Due to its inhibitory properties, in this experiment verapamil was used as a standard chemical compound. Some of synthetic (verapamil and piracetam) and herbal substances (quercetin, codeine, cyclosporine A) were purchased from Sigma-Aldrich Chemical Company (USA), while phenobarbital was obtained from PGF Cefarm-Poznan Sp. Z. o. o. (Poland). The rhizomes of *Curcuma longa* L., *Zingiberaceae,* were used as a standardized extracts and were purchased from Finzelberg GmbH & Co. KG (Germany). High performance liquid chromatography (HPLC), as a procedure included in "European Pharmacopoeia", was used to determine the content of active compounds of selected dry alcoholic extracts (86% curcuminoids, 7% essential oils). Designed experiment was performed on male Wistar rats (300–450 g). All tests were performed in accordance with Polish governmental regulations and in agreement with Local Ethics Committee on the Use of Laboratory Animals in Poznań, Poland (No. 16/2010). The experiment was performed at the Department of Pharmacology, Poznań University of Medical Sciences. The rats were kept in plastic cages, climate-controlled room with a 12 h light/dark cycles and full access to rat chow and tap water. Animals were divided into 8 groups from I to VIII, with 8 rats per group. For 21 days, all rats were receiving herbal extracts, synthetic and natural substances were suspended in water. Further details of the treatment are described in table 1. Group VIII was used as a control group. Rats included in group VIII were fed only standard diet. Wistar rats were decapitated two hours after the last administration

Table 1

List of individual groups of rats depending on the substances used

Group	Substance	Dose
I	Verapamil	20 mg kg^{-1} per day, *p. o.*
II	Cyclosporine A	5 mg kg^{-1} per day, *p. o.*
III	Piracetam	200 mg kg^{-1} per day, *p. o.*
IV	Phenobarbital	80 mg kg^{-1} per day, *p. o.*
V	Quercetin	500 mg kg^{-1} per day, *p. o.*
VI	Codeine	20 mg kg^{-1} per day, *p. o.*
VII	Rhizome extract of *Curcuma longa*	500 mg kg^{-1} per day, *p. o.*
VIII	None	Control

of particular substance. The obtained hippocampus were frozen in liquid nitrogen and preserved at –80°C.

Expression analysis

In this paper, we studied the effect of seven substances that may play a role in blocking functions of MDR1 (multidrug resistance protein 1), MRP1 (multidrug resistance-associated protein 1), MRP2 (multidrug resistance-associated protein 2), OATP1A4, OATP1A5 and OATP1C1 (SLC transporters). In the experiment, the total cellular RNA was isolated from rat hippocampus. The RNA was isolated using TriPure Isolation Reagent from Roche (Germany). BioPhotometer by Eppendorf (USA) was used to determinate the RNA's concentration and purity. According to manufacturer's protocol, complementary DNA was synthesized from 1 μg of RNA in volume of 20 μl. To obtain cDNA we used Transcriptor cDNA First Strand Synthesis Kit and oligo(dT)18 primer by Roche. According to manufacturer's protocol, LightCycler® 480 instrument (Roche) and a LightCycler®480 SYBR Green I Master (Roche) were used to carry out the real-time PCR (RT-PCR). We confirmed the amplicon size and specificity of the reaction by agarose electrophoresis and melting curve analysis. For normalization, as a housekeeping gene (endogenous internal standard), we used GAPDH cDNA. The primers and the conditions of reactions were used according to Mrozikiewicz *et al.* [17]. The LightCycler®480 Basic Software by Roche was used to evaluate data (Roche Applied Science, Berlin, Germany).

Statistical analysis

To express the mRNA content of the examined genes, mean±SEM was used. Obtained data were analysed using the SPSS 17.0 for Windows software. We compared the mean values to means of the one-way ANOVA test. As the statistically significant value, we considered the value of $p<0.05$

RESULTS

The aim of this paper was to analyse the influence of natural, synthetic substances and also herbal extracts on the level of gene expression that code transporters involved in BDB and MDR (multidrug resistance).

The study's results showed that administration of codeine and also piracetam caused a decrease of the expression in all investigated genes encoding transporters aside from Oatp1c1. Administration of cyclosporin A significantly decreased the mRNA level of Mdr1a, Mrp1 and Mrp2. We also noticed reduction of expression level for Oatp1a4, but it was not a significant decrease. Verapamil as an inhibitor of xenobiotic transporters decreased the expression level in all examined genes. In contrary to verapamil, administration of phenobarbital

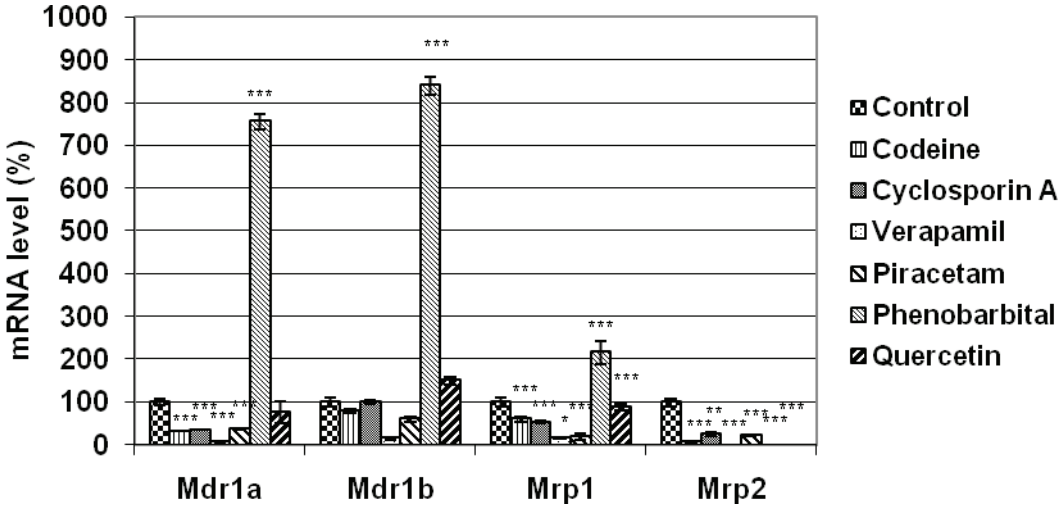

Figure 1

The expression levels of genes encoding transmembrane transporters (Mdr1a, Mdr1b, Mrp1, Mrp2) in the rat's hippocampus after 21 days of administration of synthetic and natural substances. Control groups are defined as 100%. Data are presented as mean ±SEM. Asterisks indicate significance (one-way ANOVA test): * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

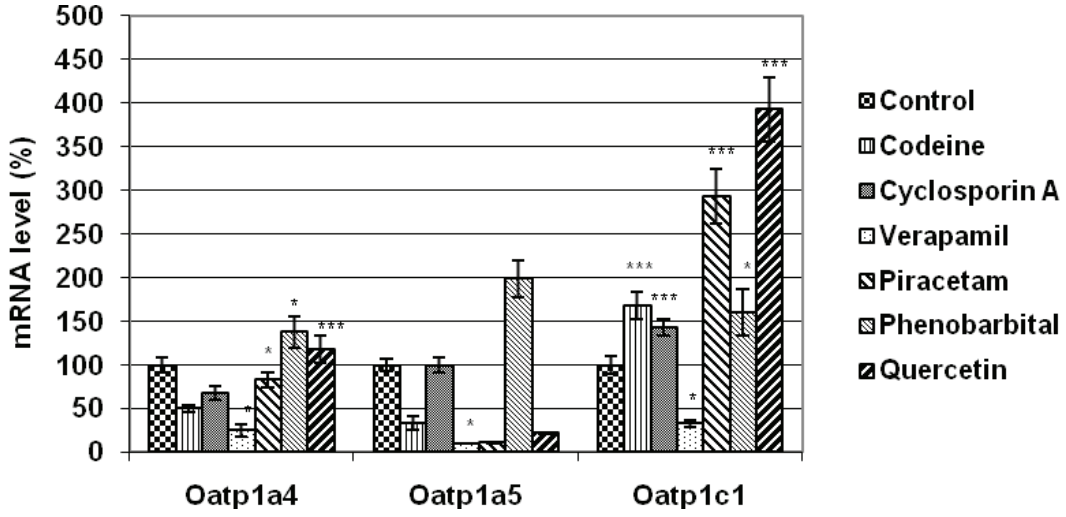

Figure 2

The expression levels of genes encoding transmembrane transporters (Oatp1a4, Oatp1a5, Oatp1c1) in the rat's hippocampus after 21 days of administration of synthetic and natural substances. Control groups are defined as 100%. Data are presented as mean ±SEM. Asterisks indicate significance (one-way ANOVA test): * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

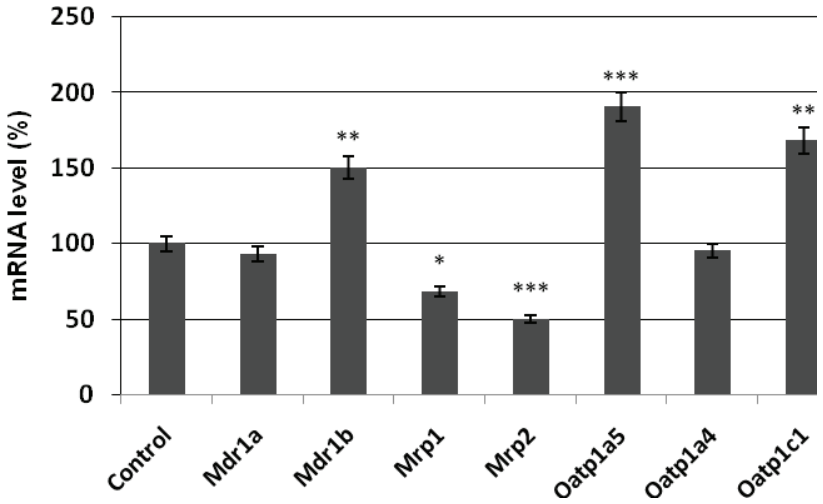

Figure 3

The expression levels of genes encoding transmembrane transporters (Mdr1a, Mdr1b, Mrp1, Mrp2, Oatp1a4, Oatp1a5, Oatp1c1) in the rat's hippocampus after 21 days of administration of *Curcuma longa* extract. Control groups are defined as 100%. Data are presented as mean ±SEM. Asterisks indicate significance (one-way ANOVA test): * $p<0.05$, ** $p<0.001$, *** $p<0.0001$

resulted in an increased expression level of all investigated genes except for Mrp2. Quercetin had an inductive effect on the expression level for Mdr1b, Oatp1a4 and Oatp1c1 but also decreased the mRNA level for Mrp2 (fig. 1, 2).

Interesting results were obtained in the herbal extract. The study showed that *C. longa* increased the expression level for Mdr1b (50%), Oatp1a5 (90%) and Oatp1c1 (68%). However, the administration of *C. longa* extract caused a decrease of the mRNA level for Mrp2 (50%) and Mrp1 (32%) (fig. 3).

DISCUSSION

Due to specific properties of blood-brain barrier, drug delivery in brain diseases often fails. Drug molecules fail to cross BBB due to their poor penetration or/and to the efflux mechanism of the brain. Nowadays, distribution of drugs to CNS across the BBB is one of the most challenging problems in pharmacy [19]. More and more scientists are focusing on finding perfect ways to inhibit active transporters that are involved in functioning of the BBB and MDR phenomenon [17].

Some of the researchers worked on *Panax ginseng*, *Hypericum perforatum* or *Ginkgo biloba* to show changes in mRNA level. Zhang *et al.* showed that administration of *P. ginseng* caused induction of P-gp expression in intestinal and brain endothelium [20]. Moreover, it also increased the level of Mrp2 mRNA in rat primary hepatocytes [21]. According to Mrozikiewicz *et al.*, *P. ginseng* extract may be useful in overcoming blood brain barrier and multidrug resistance phenomenon. *H. perforatum* seems to increase the expression level Mdr1a, Mdr1b in brain, liver, kidney, heart and also increases the expression level of Mrp2 in rat livers and kidneys [17, 22]. Furthermore, studies conducted on *G. biloba* showed that its extract can increase the expression level for Mdr1, Oatp1a4, Oatp1a5. What is interesting, the *G. biloba* extract decreased the mRNA level for Mrp2, which may be helpful in CNS diseases with treatment based on substrates for Mrp2 [16, 17, 23].

In this study, the influence that *C. longa* may have on the mRNA levels for particular transporters was analysed. The results suggests that using *C. longa* extract in the treatment of brain diseases would be effective in case of drugs which are substrates for Mrp2 and Mrp1. Yuan et al. studied mechanisms underlying subarachnoid hemorrhage (SAH) and examined how treatment based on *C. longa* extract may ameliorates SAH-induced brain oedema and BBB permeability changes. According to Yuan *et al.*, curcumin may inhibit matrix metallopeptidase-9 expression. Decreased expression of metallopeptidase-9 reduces brain oedema and attenuates post-SAH BBB disruption in mice [24].

Wang *et al.* examined if *C. longa* extract has any effect on the disruption of BBB which may be induced by brain ischaemia. Oxygen glucose deprivation(OGD)-induced disruption of paracellular permeability was weaken by *C. longa* extract. The extract also increased the expression of heme oxygenase-1 (HO-1) protein in rat brain microvascular endothelial cells (RBMECs). According to this paper, *C. longa* extract protects RBMECs against OGD-induced dysfunction of BBB thanks to the HO-1 pathway. It may mean that, under ischaemic conditions, the herbal extract may be capable of improving the barrier function of BBB. What is also interesting, that beneficial effect can be reversed by a HO-1 inhibitor [25].

Klinger and Mittal have a different opinion about *C. longa*. They wondered if *C. longa* can have any therapeutic potential during brain tumours treatment. Due to its antioxidant, anti-inflammatory, and antiproliferative properties, *Curcuma longa* is a promise in pharmacy. They focused on therapeutic features of *Curcuma longa,* maintaining that distribution of the extract to the brain might be hindered by limited BBB permeability. They showed that only adding particular nanoparticle formulations, e.g. poly(lactic-co-glycolic acid), may increase its distribution to the brain tissue [26].

CONCLUSION

Overcoming blood brain barrier to deliver drugs is still a challenge for modern scientists. As the studies show, herbal origin and synthetic drugs can modulate expression of transporters in BBB. The synthetic substance that decreased the mRNA level most was verapamil. The herbal extract we examined was obtained from *C. longa* and also reduced the mRNA level but only for Mrp2 (50%) and Mrp1 (32%). Those results might suggest that verapamil is a better inhibitor and should be used in overcoming BBB. But we should take into account that using herbal extract does not come with side effects as it comes with verapamil. In conclusion, using herbal extracts may have an interesting potential in pharmacotherapy of brain diseases.

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